Case 4-18634/A/CCN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE US PATENT APPLICATION OF
YIQING ZOU ET AL.
SERIAL NO. 07/714,229
FILED: JUNE 12, 1991
FOR: ANTIMALARIAL COMPOSITIONS

Commissioner of Patents and Trademarks
Washington D.C. 20231 USA

DECLARATION OF WALTHER H. WERNSDORFER

I, Walther H. Wernsdorfer, citizen of the Federal Republic of Germany and resident of Vienna, Austria, do hereby declare and say as follows:

That I am a Graduate of The Friedrich Alexander University of Erlangen, Federal Republic of Germany, where I graduated in 1952 and obtained the approbation in medicine (M.B.B.S);

That I am a Graduate of The Ludwig Maximilian University of Munich, Federal Republic of Germany, where I graduated in 1953 and obtained the Degree of a Doctor of Medicine (M.D.);

That I have undergone postgraduate training in tropical medicine at the Swiss Tropical Institute in Basel, Switzerland, and obtained in 1952 the Diploma of Tropical Medicine (D.T.M.);

That I have undergone postgraduate training in public health at the University of Bristol, U.K., and obtained in 1967 the Diploma of Public Health (D.P.H.);

That, as from 1958 until 1988, I have served the World Health Organization as a staff member in the fields of tropical medicine and malaria; between 1978 and 1988 as Chief Medical Officer in charge of global malaria research and *ex officio* Secretary of the Scientific Working Groups on the Chemotherapy and Immunology of Malaria, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases;

That, as from 1960, I held academic teaching assignments in addition to my WHO assignments, with the Faculty of Medicine, University of Khartoum, Sudan, the University of Tunisia, and the Université Claude Bernard, Lyon, France, where I continue to be involved in postgraduate medical training in tropical medicine;

That, in 1988, I have been appointed visiting professor at the University of Vienna, Austria, and the Universiti Sains Malaysia, Penang;

That I am the principal author or coauthor of approximately 100 publications, mainly in the field of malaria and malaria chemotherapy;

That I am a registered member of the medical profession (Medical Board of Central Franconia, Federal Republic of Germany);

That I am a member of the following professional bodies/organizations:

World Health Organization (WHO) Expert Panel on Malaria
German Society of Tropical Medicine (Honorary Member)
Swiss Society of Tropical Medicine and Parasitology (Honorary Member)
Austrian Society of Tropical Medicine and Parasitology
Royal Society of Tropical Medicine and Hygiene (U.K.)
British Society of Public Health
British Society of Parasitology;

That I am presently working as Visiting Professor (Tropical Medicine) at the Institute for Specific Prophylaxis and Tropical Medicine, Faculty of Medicine, University of Vienna, Austria, and Visiting Professor at the National Drug Research Institute, Universiti Sains Malaysia, Penang, Malaysia (Tropical Clinical Pharmacology);

That upon my request and under my guidance experimental studies on the *in vitro* activity of anti-malarial agents in *Plasmodium falciparum* have been performed at the Laboratory of Parasitology, Roslagstull Hospital for Infectious Diseases, Karolinska Institute, Stockholm, Sweden, and that I have checked for adequacy and correctness the data obtained from those tests;

That the results obtained from said studies and the conclusions drawn therefrom are the following:

1. Materials and Methods

1.1 The agents artemether (A) and benflumetol (B) have been tested *in vitro* in a 48-hours standardized exposure test in continuous culture of *Plasmodium falciparum* by measuring growth inhibition. This method is a modification of that originally described by W.H.G. Richards and B.K. Maples (Annals of Tropical Medicine and Parasitology 73: 99-108, 1979) and is summarized here as follows:

> To a viable stock culture of *Plasmodium falciparum* fresh human erythrocytes are added to produce a parasitaemia of approximately 0.4-0.5 %, adding also growth medium (RPMI 1640 LPLF, complete with 10 % serum) to obtain a haematocrit of approximately 5 %. Aliquots of 100 µl of the erythrocyte-medium mixture are pipetted into the wells of sterile, flat-bottom microtiter plates (8 x 12). Appropriate concentrations of the drug are added as standardized micro-inocula; in the control wells only the diluent is added. The dosed plates are gently agitated in order to homogenize the drug-erythrocyte-medium mixture, and then incubated at 37.5°C in a CO_2-enriched and O_2-reduced atmosphere (candle jar or CO_2 incubator). After 48 hours the plates are removed from incubation. Thin blood films are made from the sediment, i.e. erythrocyte layer, of all wells and appropriately marked. The number of parasitized erythrocytes is then counted against the total number of erythrocytes and the percentage of parasitaemia calculated. The parasitaemia in the control wells is the base parameter for optimum growth, and the relative inhibition in the drug wells calculated accordingly.
>
> Drug-induced growth inhibition is gradual according to the drug concentration, usually following a logdose-normal (Gaussian) distribution.

1.2 The agent A has been tested in triplicate against *Plasmodium falciparum* isolates T 994 (from Thailand) and LS 21 (from India) over the concentration range of 10^{-12} to 10^{-5} mol/l. Similarly, agent B has been tested in triplicate with both isolates over the concentration range of 2×10^{-12} to 2×10^{-5} mol/l.
Isolate T 994 is highly resistant to chloroquine and pyrimethamine. Isolate LS 21 is moderately resistant to both compounds.

1.3 The agents A and B have been tested together in chess board design against *Plasmodium falciparum* isolates T 994 and LS 21 over the concentration range of 10^{-11} to 10^{-7} mol/l, applying duplicate tests.

2. Results

2.1 The standard logdose-probit regressions according to B. Grab and W.H. Wernsdorfer (WHO document WHO/MAL/83.990, 1983) are shown separately for agents A and B in Fig. 1 and Fig. 2. In this system the concentrations are plotted on the x-axis according to a logarithmic scale, and the % inhibition on the y-axis according to a probit scale, as originally introduced by J.T. Lichtfield and F. Wilcoxon (Journal of Pharmacology and Experimental Therapy 96; 99-113, 1949) and subsequently accepted throughout the world in the analysis of sensitivity tests. The regression lines in Fig. 1 and Fig. 2 show a good fit of observed to expected data points, with

$\chi^2 = 0.52656$ for agent A in isolate T 994 (maximum permissible 11.5)
$\chi^2 = 0.07944$ for agent A in isolate LS 21 (maximum permissible 11.5)
$\chi^2 = 0.74670$ for agent B in isolate T 994 (maximum permissible 9.49)
$\chi^2 = 1.00479$ for agent B in isolate LS 21 (maximum permissible 9.49).

2.2 There is indication of enhanced antimalarial interaction between the two agents over the concentration range tested. It is quite pronounced with both *Plasmodium falciparum* isolates at molecular ratios of A:B = 1:1 up to 1:30, and most marked at a ratio A:B = 1:3 with isolate T 994 and A:B = 1:10 with isolate LS 21.

The antimalarial interaction increases with the inhibitory concentration level (EC), being most pronounced at the EC_{99}, i.e. the concentration required for 99 % inhibition of parasite reproduction (see Table 1 (Exhibit 1) and Fig. 3-5 for isolate T 994 and Table 2 (Exhibit 2) and Fig. 6-8 for isolate LS 21). Here the observed inhibition is between 25 and 200 times higher than the expected inhibition as calculated from the inhibitory profiles of the monosubstances based on a merely additive effect.

The expected numerical values in Table 1 and Table 2 have been calculated from the geometrical mean, not from the arithmetical mean of the observed individual values. Calculating the geometrical mean is appropriate to the logdose system employed and to substances with different concentration response. It permits also the determination of expected values for drug mixtures of various proportions. Unlike the arithmetical mean it is therefore not restricted to mixtures in the proportion 1:1.

The activity of the combined agents at the various molecular ratios shows a good fit of the observed data to the mathematically derived regression lines (Table 3 - Exhibit 3).

The differences between the expected and the observed inhibition at EC_{50}, EC_{90} and EC_{99} level are significant throughout at A:B ratios of 1:1 to 1:30 with both isolates.

2.3 The following figures (Exhibits 4-11) are part of this Declaration:

Fig.1: Growth inhibition of *Plasmodium falciparum* isolates T 994 and LS 21 at various concentrations of artemether (logdose-probit regression)

Fig.2: Growth inhibition of *Plasmodium falciparum* isolates T 994 and LS 21 at various concentrations of benflumetol (logdose-probit regression).

Fig.3: Growth inhibition of *Plasmodium falciparum* isolate T 994 by various concentrations of artemether + benflumetol at the molecular ratio of 1:1. Expected regression as represented by dashed line (- - - - -) based on additive effect.

Fig.4: Growth inhibition of *Plasmodium falciparum* isolate T 994 by various concentrations of artemether + benflumetol at the molecular ratio of 1:3. Expected regression as represented by dashed line (- - - - -) based on additive effect.

Fig.5: Growth inhibition of *Plasmodium falciparum* isolate T 994 by various concentrations of artemether + benflumetol at the molecular ratio of 1:10. Expected regression as represented by dashed line (- - - - -) based on additive effect.

Fig.6: Growth inhibition of *Plasmodium falciparum* isolate LS 21 by various concentrations of artemether + benflumetol at the molecular ratio of 1:1. Expected regression as represented by dashed line (- - - - -) based on additive effect.

Fig.7: Growth inhibition of *Plasmodium falciparum* isolate LS 21 by various concentrations of artemether + benflumetol at the molecular ratio of 1:3. Expected regression as represented by dashed line (- - - - -) based on additive effect.

Fig.8: Growth inhibition of *Plasmodium falciparum* isolate LS 21 by various concentrations of artemether + benflumetol at the molecular ratio of 1:10. Expected regression as represented by dashed line (- - - - -) based on additive effect.

2.4 The observed and expected EC_{50}, EC_{90} and EC_{99} levels and their confidence limits in *Plasmodium falciparum* exposed to mixtures of artemether + benflumetol at various molecular

ratios are presented in Table 1 for isolate T 994, and in Table 2 for isolate LS 21. The goodness of fit of the observed vs expected data at various molecular ratios of artemether and benflumetol are shown in Table 3.

3. Conclusion

There is evidence of enhanced interaction between components A and B against *Plasmodium falciparum in vitro* at molecular ratios A:B = 1:1 to 1:30. Over this range the EC_{50}, EC_{90} and EC_{99} concentrations were consistently and significantly lower than those expected if the interaction were only additive. At the EC_{99} the combined effect was most marked, the observed EC_{99} concentrations being only 1/25 to 1/214 of those expected with merely additive activity. This is clearly an indication of true synergism and would explain the therapeutc results where the components A and B alone showed substantial failure rates while together they produced practically universal cure. Clinical pharmacological investigations indicate that combined oral treatment at the recommended doses produces molecular A:B ratios in the blood which are within the range of A:B = 1:3 to 1:30 for most of the time.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Signed at Vienna, Austria, this ...9th... day of May 1993

Walther H. Wernsdorfer

11 Exhibits are part of this Declaration:

Exhibits 1-3: 3 Tables
Exhibits 4-11: 8 Figures

Exhibit 1

Table 1: Observed and expected EC_{50}, EC_{90} and EC_{99} levels in *Plasmodium falciparum* isolate T 994 exposed to mixtures of artemether and benflumetol at various molecular ratios (concentrations expressed as nmol/l)

Level	Parameter	A Alone	B Alone	A + B Mixture (combined concentration)				
				1 : 1	1 : 2	1 : 3	1 : 10	1 : 30
EC_{50}	Observed	0.5730	2.0948	0.2980	0.3743	0.3379	0.6273	1.1458
	LCL*	0.4350	1.6026	0.2468	0.2798	0.2785	0.5001	0.9021
	HCL**	0.7547	2.7382	0.3598	0.5007	0.4100	0.7869	1.4554
	Expected	-	-	1.0956	1.3598	1.5149	1.8619	2.0090
	LCL*	-	-	0.6971	1.0376	1.1568	1.4234	1.5366
	HCL**	-	-	1.4375	1.7820	1.9840	2.4355	2.6267
	Obs vs Exp.	-	-	Sign.	Sign.	Sign.	Sign.	Sign.
EC_{90}	Observed	113.2474	246.5273	5.3413	12.9471	5.3736	13.8243	19.7951
	LCL*	71.4375	149.8614	3.8532	8.3798	3.8568	9.8459	14.6107
	HCL**	179.5273	405.5461	7.4040	20.0037	7.4870	19.4102	26.8192
	Expected	-	-	167.0885	190.2185	202.9578	229.6955	240.4180
	LCL*	-	-	103.4685	117.0672	124.5228	140.1001	146.3222
	HCL**	-	-	269.8270	309.0796	330.7979	376.5881	395.0243
	Obs vs Exp.	-	-	Sign.	Sign.	Sign.	Sign.	Sign.
EC_{99}	Observed	8433.5936	12029.152	56.1878	232.8042	51.2745	172.1032	202.0758
	LCL*	4042.7919	5506.965	33.1515	113.5640	29.7546	97.3231	120.9659
	HCL**	17593.1639	26275.903	95.2316	477.2442	88.3586	304.3421	337.5715
	Expected	-	-	10072.189	10686.305	11007.265	11647.019	11892.142
	LCL*	-	-	4718.423	4967.852	5097.469	5354.384	5452.332
	HCL**	-	-	21500.611	22987.219	23768.635	25334.952	25938.084
	Obs vs Exp.	-	-	Sign.	Sign.	Sign.	Sign.	Sign.

* LCL = Lower confidence limit AT $p < 0.05$

** HCL = Higher conficence limit AT $p < 0.05$

Exhibit 2

Table 2: Observed and expected EC_{50}, EC_{90} and EC_{99} levels in *Plasmodium falciparum* isolate LS 21 exposed to mixtures of artemether and benflumetol at various molecular ratios (concentrations expressed as nmol/l)

Level	Parameter	A Alone	B Alone	A + B Mixture (combined concentration)				
				1 : 1	1 : 2	1 : 3	1 : 10	1 : 30
EC_{50}	Observed	0.2284	3.6074	0.2739	0.4103	0.3699	0.8664	1.5714
	LCL*	0.1818	2.7450	0.2281	0.3105	0.2922	0.7043	1.2369
	HCL**	0.2869	4.7409	0.3290	0.5423	0.4682	1.0657	1.9962
	Expected	-	-	0.9077	1.4378	1.8095	2.8070	3.3001
	LCL*	-	-	0.7064	1.1106	1.3925	2.1447	2.5148
	HCL**	-	-	1.1663	1.8613	2.3514	3.6739	4.3308
	Obs vs Exp.	-	-	Sign.	Sign.	Sign.	Sign.	Sign.
EC_{90}	Observed	10.5360	424.4362	4.3503	11.9973	13.1133	15.2016	31.8761
	LCL*	7.4032	251.8600	3.1702	7.8793	9.0615	11.0367	22.6253
	HCL**	14.9944	715.2628	5.9697	18.2674	18.9768	20.9381	44.9093
	Expected	-	-	66.8720	123.8132	168.4722	303.3129	376.7331
	LCL*	-	-	43.1807	77.7283	104.2856	182.7722	224.7751
	HCL**	-	-	103.5613	197.2211	272.1645	503.3515	631.4214
	Obs vs Exp.	-	-	Sign.	Sign.	Sign.	Sign.	Sign.
EC_{99}	Observed	239.5630	20706.238	41.4631	188.0854	240.5788	157.1631	370.9305
	LCL*	138.5049	9175.355	24.8789	94.7098	128.952	93.3270	206.7107
	HCL**	414.3566	46928.252	69.1023	393.5212	448.8236	264.6632	665.6133
	Expected	-	-	2227.206	4683.178	6790.955	13805.090	17931.953
	LCL*	-	-	1127.312	2267.620	3216.129	6267.049	8014.490
	HCL**	-	-	4400.245	9671.885	14339.308	30409.928	40121.701
	Obs vs Exp.	-	-	Sign.	Sign.	Sign.	Sign.	Sign.

* LCL = Lower confidence limit AT $p < 0.05$

** HCL = Higher conficence limit AT $p < 0.05$

Exhibit 3

Table 3: Goodness of fit of logdose-probit regressions from growth inhibition tests in *Plasmodium falciparum* isolates T 994 and LS 21 *in vitro*, using artemether and benflumetol together at various molecular ratios

Isolate	Compound	Molecular	χ^2 for Heterogeneity	
			Observed	Maximum Permissible
T 994	A + B	1 : 1	0.10306	11.1
T 994	A + B	1 : 2	0.01816	7.82
T 994	A + B	1 : 3	0.12156	9.49
T 994	A + B	1 : 10	0.10716	9.49
T 994	A + B	1 : 30	0.08388	7.82
LS 21	A + B	1 : 1	0.09728	11.1
LS 21	A + B	1 : 2	0.08708	7.82
LS 21	A + B	1 : 3	0.13766	11.1
LS 21	A + B	1 : 10	0.21790	9.49
LS 21	A + B	1 : 30	0.46246	7.82

Exhibit 4

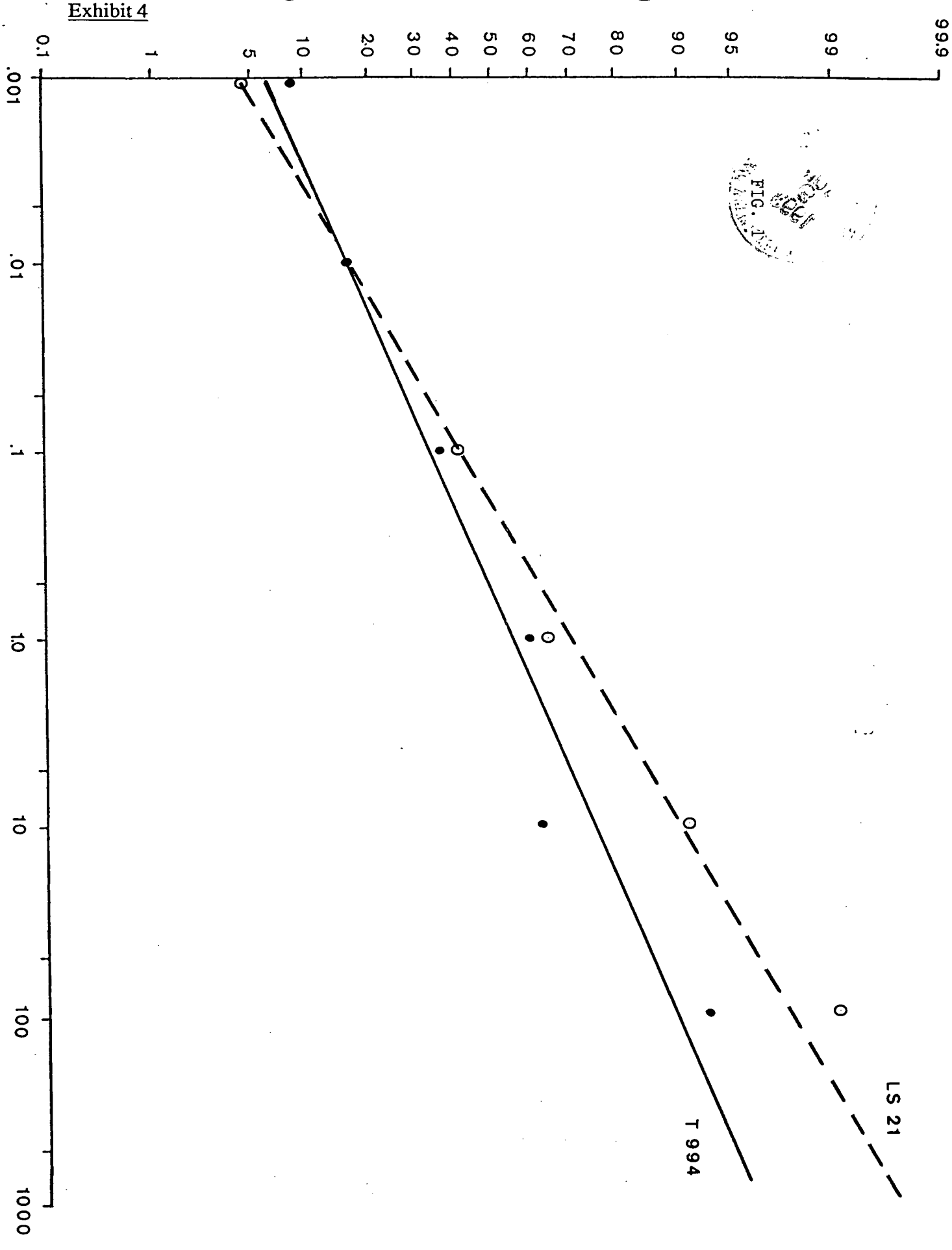


FIG. 1

Exhibit 5

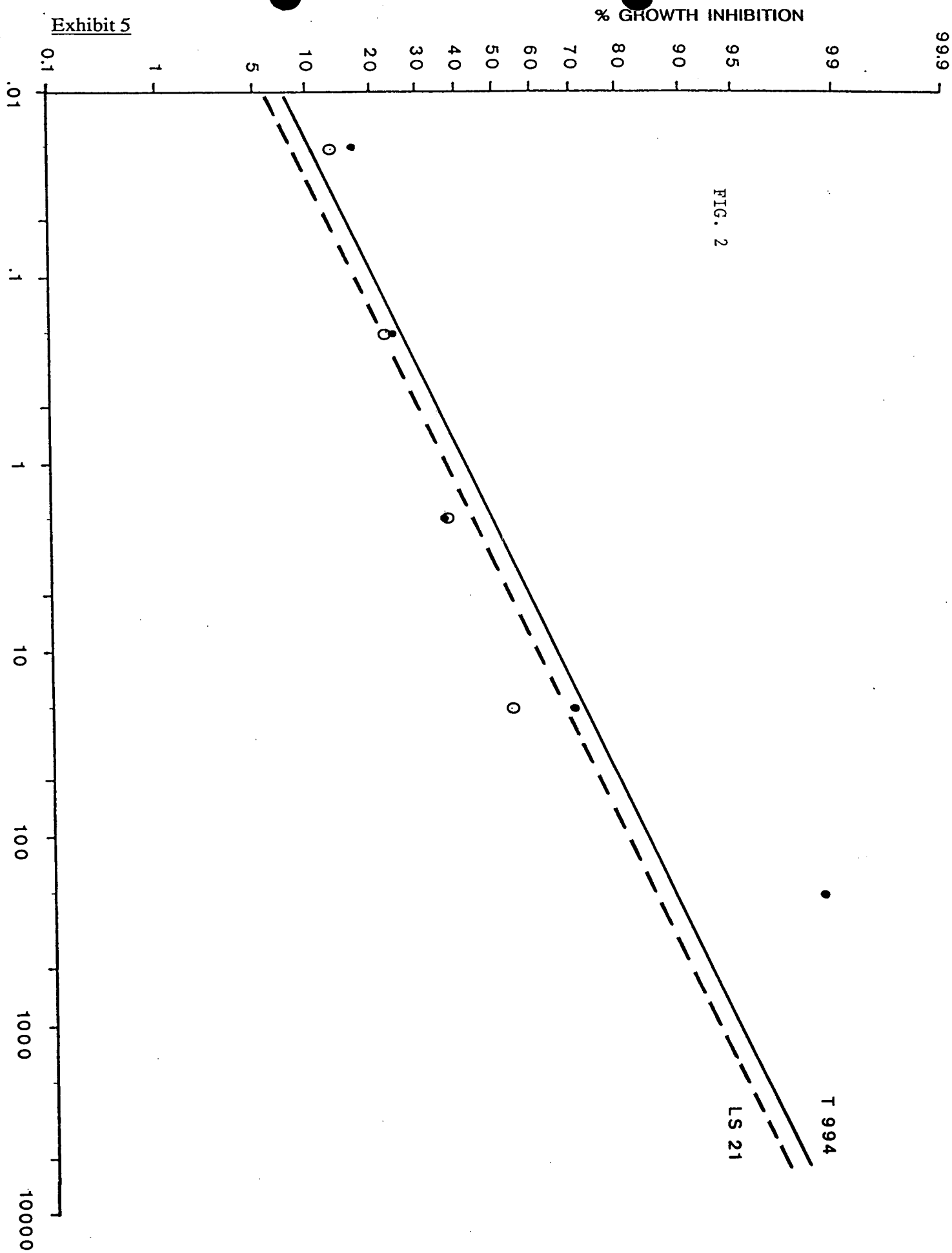


FIG. 2

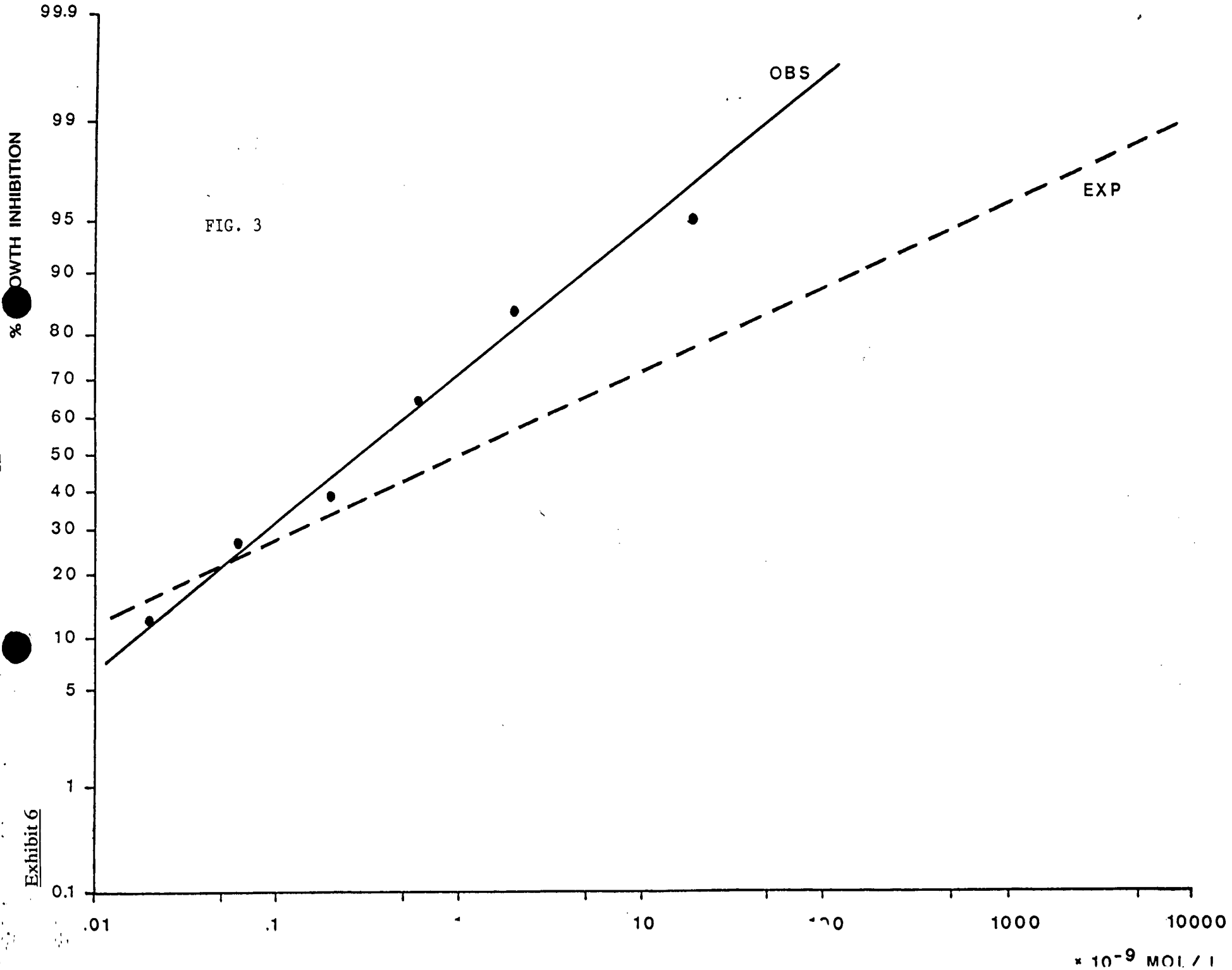


FIG. 3

Exhibit 6

Exhibit 7

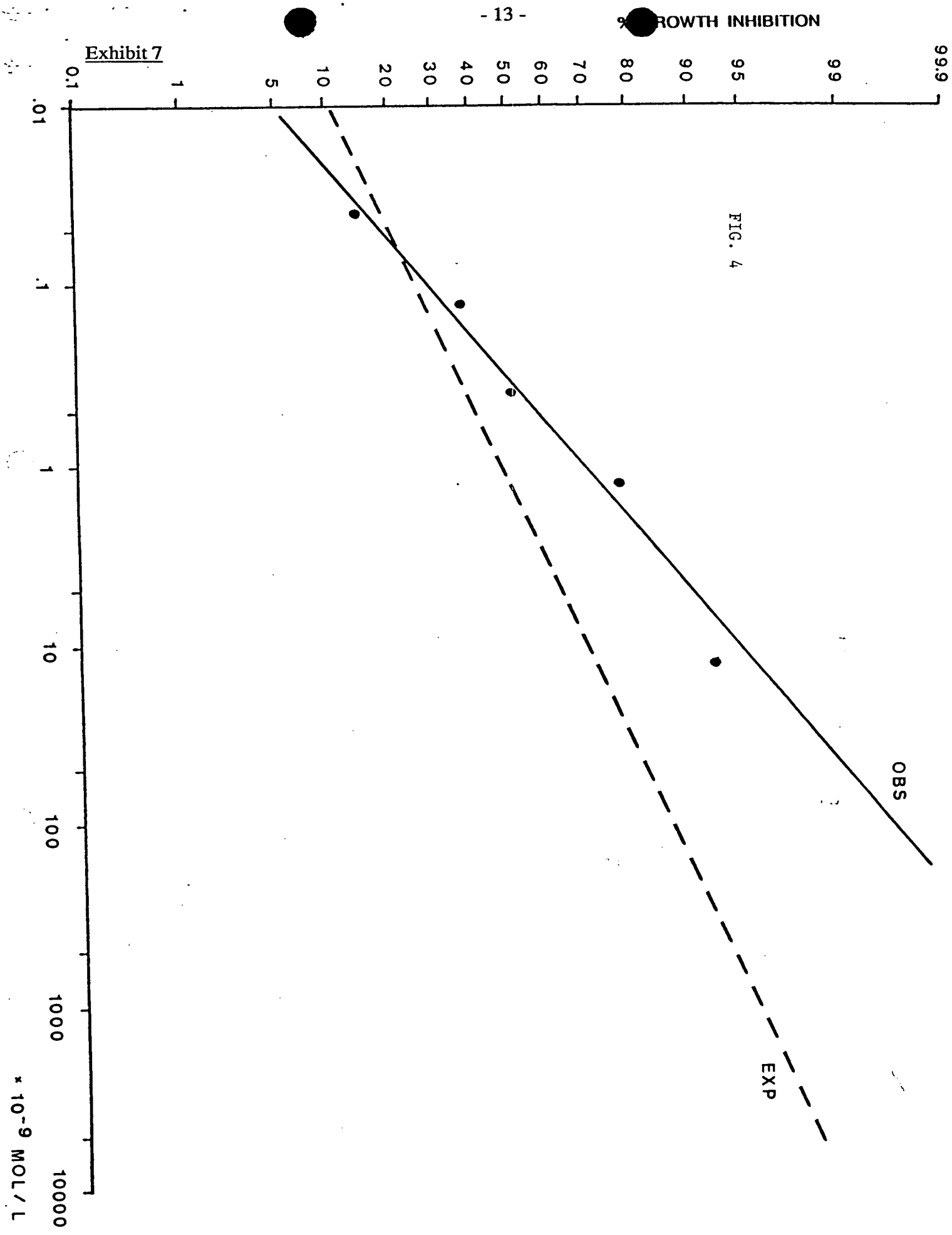
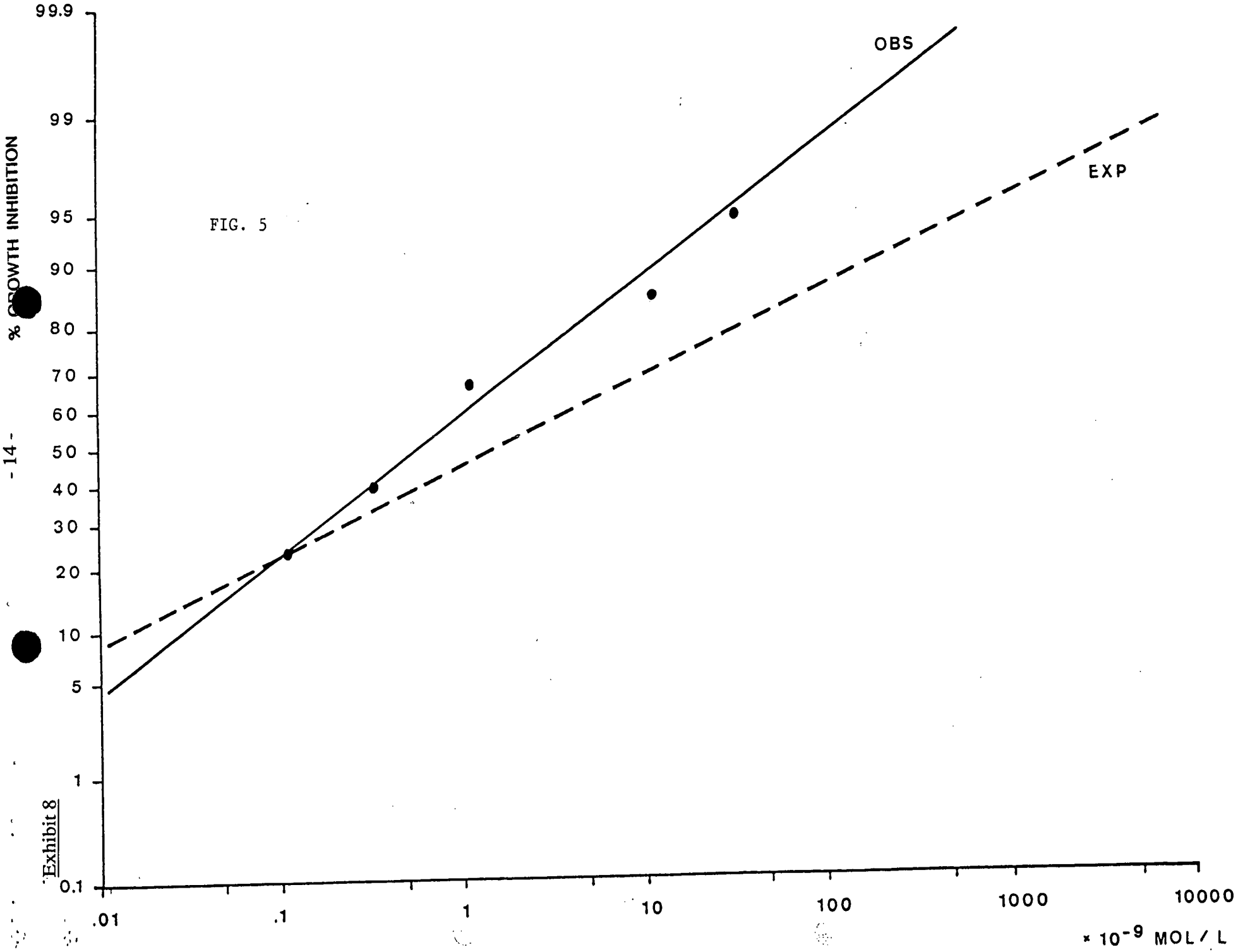


FIG. 4

FIG. 5



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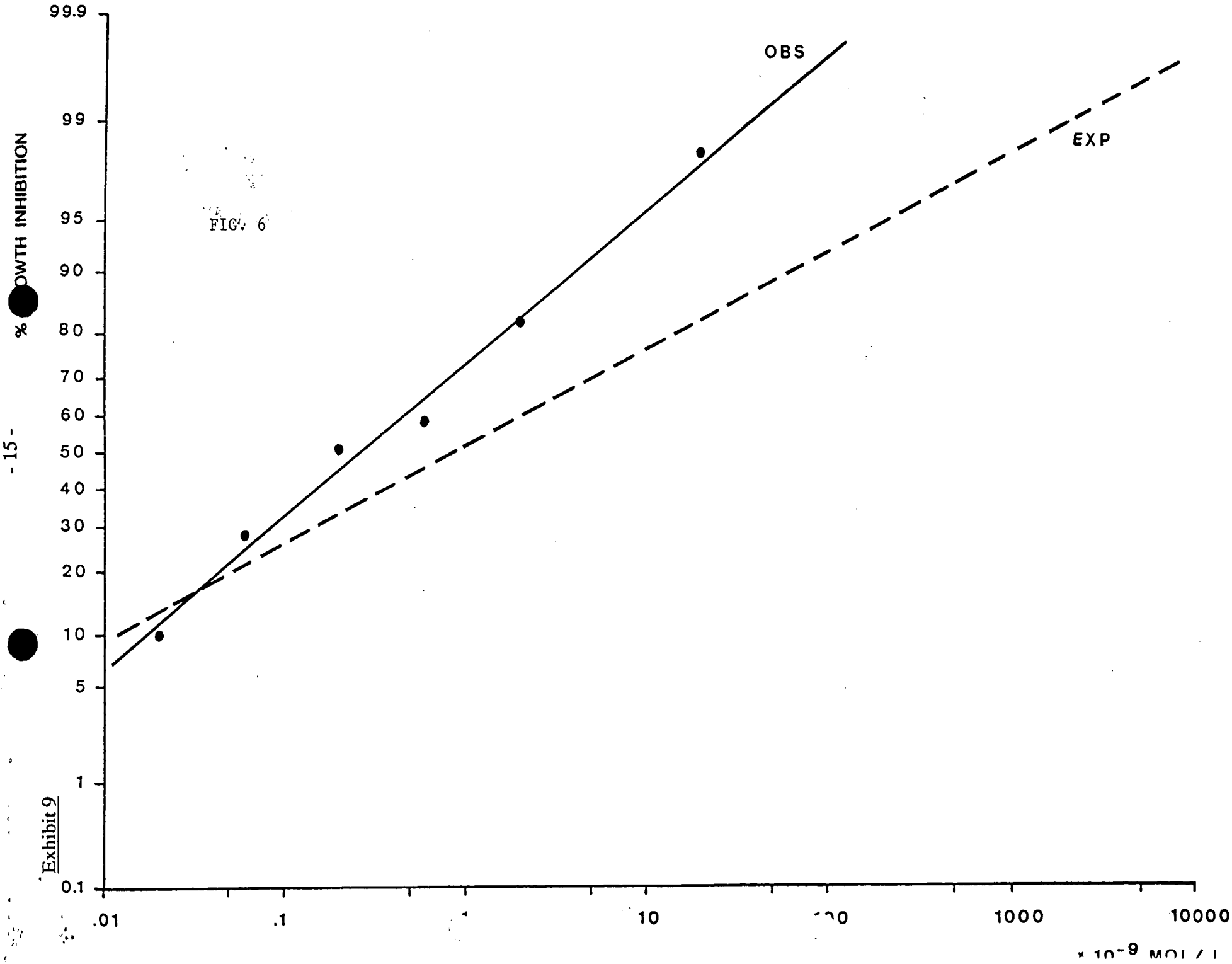

FIG. 6

Exhibit 9

FIG. 7

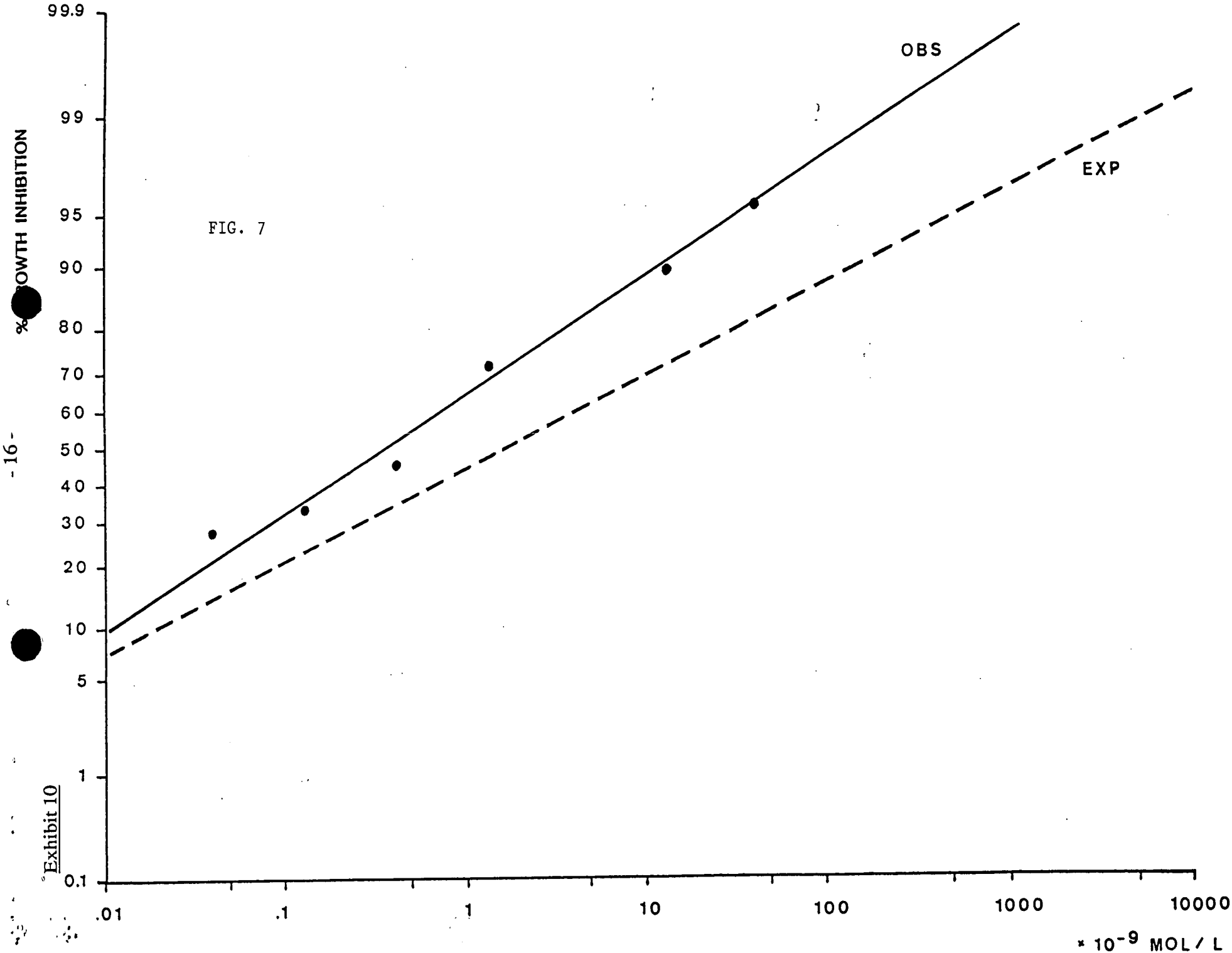

Exhibit 10

FIG. 8

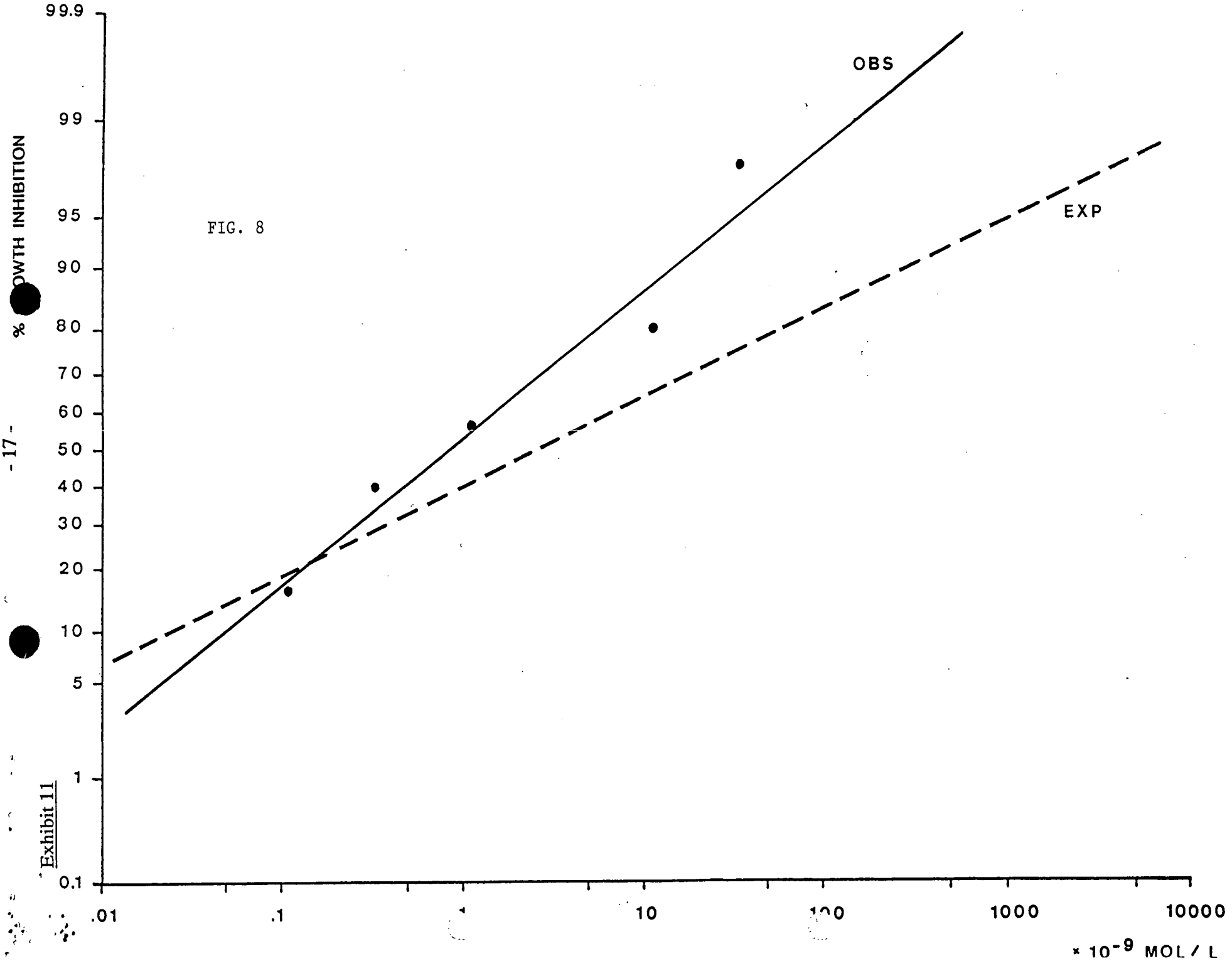

Exhibit 11